

HPLC ANALYSIS, ANTIMICROBIAL AND ANTIFUNGAL ACTIVITY OF AN EXPERIMENTAL PLANT BASED GEL, FOR ENDODONTIC USAGE

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ABSTRACT. The aim of this study is to investigate the active compounds of an experimental gel and to evaluate its antimicrobial and antifungal effects. This gel consists of a mix extract of *Arctium lappa* L. root powder and *Aloe vera* (L.) Burm.f. gel and is used in endodontic procedures. With the help of HPLC/ESI(+)-MS method, various active compounds were identified in the experimental extract. For the evaluation of the antimicrobial and antifungal activity, microorganisms commonly found in endodontic infections were used. The agar-diffusion method was performed for each plant formula and the microdilution method was used to obtain the minimum inhibitory concentration and also the minimum bactericidal concentration. Clear inhibition zones around discs indicated the presence of antimicrobial and antifungal activity for the disc diffusion assay. The minimum inhibitory and bactericidal concentration obtained were compared to the antibiotics or antifungals, specific for each microorganism.

Keywords: antimicrobial, antifungal, HPLC/ESI(+)-MS, agar-diffusion, microdilution, *Arctium lappa*, *Aloe vera*

INTRODUCTION

***Arctium lappa* L.**, also known as burdock, is a plant belonging to the Compositae (Asteraceae) family, commonly found in Europe, North America and Asia [1]. In traditional medicine, burdock is famous for a wide variety of benefits. It has the capacity to reduce the amount of fluids in the organism, to treat skin diseases, for example eczema, acne or psoriasis, and to purify blood [2][3]. In Europe the plant is wide spread, except for the extreme northern regions of the continent [4].

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Previous studies linked *Arctium lappa* to multiple therapeutic activities. It has a stronger antioxidant effect than other vegetables or fruits [5]. Chemical reactions with free-radical terminations and oxygen inhibitory compounds found in *Arctium lappa* are accounting for suppressing biochemical processes promoted by oxygen [6].

Studies regarding the antidiabetic properties of burdock roots demonstrated that phenolic compounds control the glycemic and lipidic level and are helpful in avoiding complications of the disease. Burdock roots can be used in food and beverages, as additional products to classic antidiabetic drugs [7].

Continuous development of anticancer medications, with minimum side effects, led to investigations of plant based products. On account of the antiproliferative effects of burdock, the plant extracts are able to exhibit selective antitumoral activity against leukaemia, kidney and mammary human cancer cell lines [8].

An important antimicrobial effect was also assigned to *Arctium lappa*. Research showed that the root of burdock possesses antimicrobial properties against food-related microorganisms, like *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus subtilis*, *Escherichia coli*, *Shigella dysenteriae* and *Salmonella typhimurium* [9].

***Aloe vera* (L.) Burm.f.**, is a plant belonging to the ample genus *Aloe*, of which many species are cultivated all over the world. It is used in a diversity of fields, from a therapeutic agent, due to its anti-inflammatory, antimicrobial and antifungal properties, to a decoration plant in the Mediterranean regions [10].

Products obtained from *Aloe vera* have global applications, ranging from the food industry to the pharmaceutical and cosmetic industry [11].

In the food industry the plant is used in numerous products, especially for the preparation of diets with a content of biologically-active compounds [12]. Tonic syrups and other refreshment drinks are obtained from the gel of this plant [13].

Aloe vera extracts are as well used in the pharmaceutical and cosmetic fields. Studies on hairless mice proved that oral administration of *Aloe vera* gel powder can prevent the loss of skin suppleness and can postpone the aging of the dermis caused by exposure to ultraviolet B radiations [14]. Skin dehydration is as well avoided with the help of polysaccharide-rich elements of the plant, which are responsible for increasing the content in water of the stratum comeum [15].

Medicinal tablets with particular roles were produced. Pills containing *Aloe vera* were conceived for enhancing the absorption of vitamins C and E [16] and tablets coated with *Aloe vera* gel were used for the controlled release of active substances [17].

Extracts of this plant can be valuable treatments for burn injuries, by minimizing the healing time [18][19] and maintaining the collagen content in the primary phase of dermal healing [20].

Antimicrobial and antifungal properties of *Aloe vera* were likewise explored. *Aloe vera* products can be used as agents against *Helicobacter pylori* strains [21], against *Enterococcus bovis*, *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Morganella morganii* and *Klebsiella pneumoniae* [22] but also against fungal pathogens, like *Candida albicans* [23]. Moreover, antidiabetic [24], anticancer [25] and antiviral effects [26] of the plant encourage researchers to explore the development of its curative capacities.

Plants have high applicability not only in general medicine, but also in dental medicine. Antimicrobial effects of *Arctium lappa* in dentistry, against oral microorganisms, were investigated and proven during several studies. Burdock extracts are effective against bacteria commonly found in the oral cavity, like *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans*, *Candida albicans*, *Candida tropicalis* and *Candida glabrata* [27].

Aloe vera can be used as an oral dentifrice [28] or as a mouthwash, due to its capacity to diminish dental plaque. Equally, it prevents lesions of the oral mucosa, produced by anticancer radiation therapy [29]. Other uses in the field of periodontology are as local drugs in periodontal pockets [30] or as acemannan sponges, to prevent damage of the alveolar bone, cementum and periodontal ligament [31].

Particularly in endodontics, the necessity of plant based therapeutic agents appeared due to the side effects of the frequently used irrigants and local medicaments. Situations when sodium hypochlorite reaches the periapical tissues, beyond the apical foramen, are undesirable for every endodontist, since they can produce severe reactions. Patients can suffer pain episodes, hematoma and in rare cases even necrosis of the subcutaneous tissues [32]. Similar research showed that the chemical reaction sodium hypochlorite and chlorhexidine can produce para-chloroaniline, an organochlorine compound which exhibits a potentially carcinogenic effect [33][34].

A variety of plants, like *Caryophyllus aromaticus* L., *Glycyrrhiza glabra* L., *Camellia sinensis* (L.) Kuntze, *Apium graveolens* L., were studied as natural alternatives to the classic root canal disinfecting substances [35]. In relation to the antimicrobial effect of *Arctium lappa* and *Aloe vera* against root canal specific microorganisms, extended investigations opened new areas of interest for researchers and practitioners. Inhibitory effects were reported against bacteria like *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* or *Bacillus subtilis* and as well against yeasts, like *Candida albicans* [36][37][38][39].

RESULTS AND DISCUSSION

HPLC/ESI(+)-MS analysis of the extracts revealed a total of 12 phenolic compounds, 11 for *Arctium lappa* root extract and 1 for *Aloe vera* extract. (Fig.1) (Fig.2).

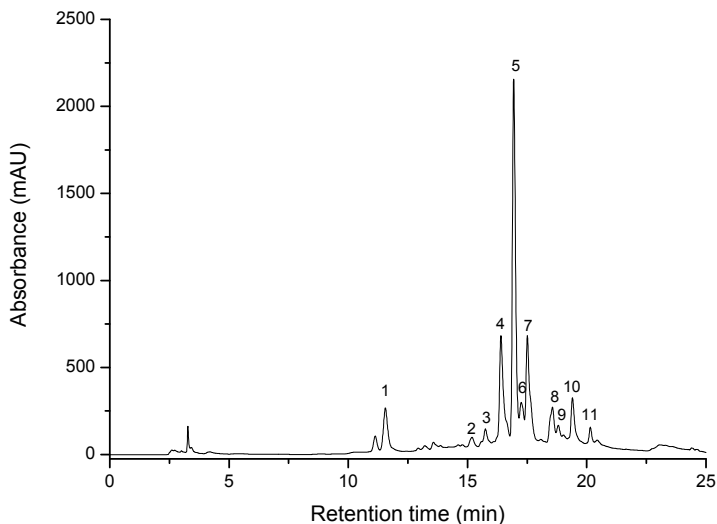


Figure 1. Chromatogram determined by HPLC /ESI(+)-MS analysis of phenolic compounds present in *Arctium lappa* root extract. For peak assignment see Table 1

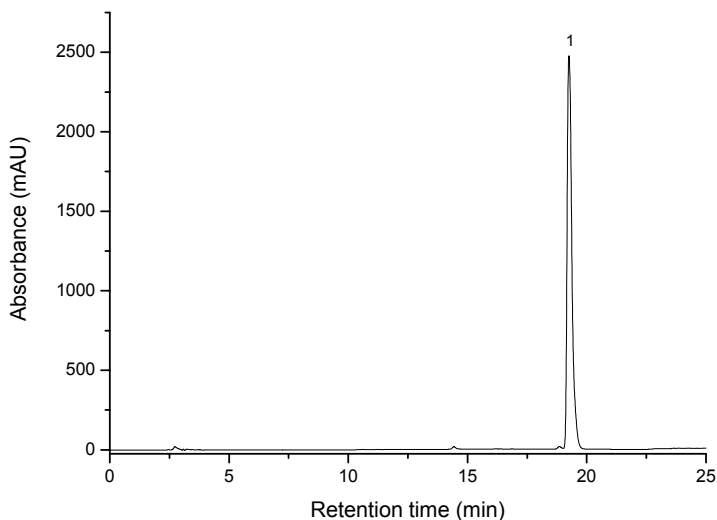


Figure 2. Chromatogram determined by HPLC /ESI(+)-MS analysis of phenolic compounds present in *Aloe vera* extract. For peak assignment see Table 2

For *Arctium lappa* root extract we identified peak 1 as chlorogenic acid (5-caffeoylquinic acid), with a $[M + H]^+$ ion at 355 m/z. Peaks 2 and 3 had $[M + H]^+$ ion at 517m/z and were identified as isomers of the caffeoylquinic acid. Chlorogenic acids are esters of the quinic acid and *trans*-cinnamic acids, such as caffeic acid, *p*-coumaric acid or ferulic acid [40]. They are studied and used for their antioxidant and antiinflammatory properties [41][42]. Antibacterial and antifungal activity of chlorogenic acids were as well reported in different studies. Attached to the external membrane of the bacteria, chlorogenic acid is able to break it and to induce cell destruction [43][44].

In endodontics, the cleaning effect of chlorogenic acid was previously observed. As an example, chamomile is able to exhibit antimicrobial activity trough the properties of chlorogenic acid and trough the properties of other phenolic components [45]. *Passiflora Edulis* is also effective in the removal of the smear layer from the root canal walls, due to its content in chlorogenic acid and other bioactive substances [46].

Peaks 4 and 5 had the same cvasimolecular ion at 537 m/z and were identified as lappaol A and isolappaol A. Lappaol F was identified at peak 7, with $[M + H]^+$ ion at 713 m/z. Peak 6 and 11, with $[M + H]^+$ ion at 373 m/z, were identified as arctiin and arctigenin. Arctignan E and Arctignan D were identified at peaks 8 and 10, with $[M + H]^+$ ion at 733 m/z, while at peak 9 we identified matairesinol, with $[M + H]^+$ ion at 359 m/z.

The major compound identified in *Arctium lappa* was arctiin and its derivatives, artignan and arctigenin. Arctiin, artignan, arctigenin and matairesinol are defined as lignan compounds, a group of phenylpropane derivatives [47] Likewise defined as lignans are lappaol A, isolappaol A and lappaol F. They are also known as sesquilignans.

Arctium lappa produces lignans as secondary metabolites, with various pharmacological properties. The antioxidant and the recent discovered anticancer effect make lignans the starting point for new drugs discovery [48][49]. Recent studies showed that lignans identified in *Arctium lappa* possess the ability to reverse multidrug resistance and even to amplify the efficacy of some drugs, while decreasing the dose needed to reach cell toxicity [50]. Lignans, and especially arctiin and its derivatives, are known for their antiinflammatory effect on macrophages, by diminishing the quantity of nitric oxide and pro-inflammatory cytokines, like TNF- α and IL6 [51][52].

The compound we identified in *Aloe vera* extract was *aloe-emodin*. It belongs to the class of anthraquinones, a group of natural phenolic substances. It is active against biofilms, especially against *Streptococcus mutans* [53], and against different Gram-positive and Gram-negative bacteria [54][55][56]. Its important activity against *methicillin-resistant Staphylococcus aureus* (MRSA) [57] and its antifungal activity against *Candida albicans* [58] were also confirmed.

Aloe-emodin was as well described as an anticancerous agent against neuroectodermal tumors [59], lung carcinoma cells [60] and hepatic cancer cell lines [61].

Table 1. Phenolics compounds in *Arctium lappa*

Peak No.	Compounds	m/z value	R _t (min)	Concentration (mg/100g)
1	Chlorogenic acid (5-caffeoylquinic acid)	355	11.55	114.529
2	Caffeoylquinic acid isomer	517	15.19	84.062
3	Caffeoylquinic acid isomer	517	15.74	95.942
4	Lappaol A	537	16.40	186.706
5	Arctiin	535, 373	16.93	323.886
6	isoLappaol A	537	17.25	104.601
7	Lappaol F	713	17.51	181.077
8	Arctignan E	733	18.56	111.676
9	Matairesinol	359	18.98	85.165
10	Arctignan D	733	19.40	133.115
11	Arctigenin	373	20.15	87.042

Table 2. Anthraquinone in *Aloe barbadensis* Miller

Peak No.	Compounds	m/z value	R _t (min)	Concentration (mg/100g)
1	Aloe-emodin	271	19.25	131.728

The agar-diffusion method was used to determine the antimicrobial and antifungal activity of the microorganisms, commonly found in endodontic infections. The results were expressed in millimetres, representing the average inhibition diameters around the discs.

Arctium lappa root formula exhibited the largest zone of inhibition for *Candida albicans* (ATCC10231), with an average diameter of 11.65 mm, followed by 3.69 mm for *Enterococcus faecalis* (ATCC29212). A smaller zone of inhibition to this formula exhibited *Pseudomonas aeruginosa* (ATCC27853), with an average diameter of 2.63 mm, *Parvimonas micra* (ATCC33270), 0.65 mm, *Peptostreptococcus anaerobius* (ATCC27337), 0.56 mm and *Fusobacterium nucleatum* (ATCC25586), 0.14 mm (Table 3).

Aloe vera gel formula exhibited the largest zone of inhibition for *Candida albicans* (ATCC10231), 11.35 mm, followed by 6.24 mm for *Enterococcus faecalis* (ATCC29212). A medium sensitivity to this formula exhibited *Fusobacterium nucleatum* (ATCC25586), 3.65 mm, *Pseudomonas aeruginosa* (ATCC27853), 3.61 mm, *Peptostreptococcus anaerobius* (ATCC27337), 2.61 mm and *Parvimonas micra* (ATCC33270), 1.56 mm (Table 3).

The experimental mix of *Arctium lappa* root and *Aloe vera* gel exhibited the largest zone of inhibition for *Candida Albicans* (ATCC10231), with an average diameter of 11.62 mm, followed by 5.69 mm for *Enterococcus faecalis* (ATCC29212). A medium sensitivity to this formula exhibited *Pseudomonas aeruginosa* (ATCC27853), with an average diameter of 3.45 mm, *Peptostreptococcus anaerobius* (ATCC27337), 2.02 mm, *Fusobacterium nucleatum* (ATCC25586), 1.89 mm and *Parvinomas micra* (ATCC33270), 1.01 mm (Table 3).

In order to compare specific antibiotics and antifungals, corresponding to the *Clinical and Laboratory Standards Institute*, inhibition zones for Amoxicillin and Clavulanic acid, Metronidazole, Ciprofloxacin and Fluconazole were determined (Table 4).

Table 3. Inhibition zones for plant extracts (mm.)

Samples	<i>P. aeruginosa</i> (ATCC 27853)	<i>E. faecalis</i> (ATCC 29212)	<i>P. micra</i> (ATCC 33270)	<i>P. anaerobius</i> (ATCC 27337)	<i>F. nucleatum</i> (ATCC 25586)	<i>C. albicans</i> (ATCC 10231)
<i>Arctium lappa</i> root extract (mg/ml)	2.63	3.69	0.65	0.56	0.14	11.65
<i>Aloe barbadensis</i> Miller gel extract (mg/ml)	3.61	6.24	1.56	2.61	3.65	11.35
Mix (mg/ml)	3.45	5.69	1.01	2.02	1.89	11.62

Table 4. Inhibition zones for standard CLSI medications (mm.)

Samples	<i>P. aeruginosa</i> (ATCC 27853)	<i>E. faecalis</i> (ATCC 29212)	<i>P. micra</i> (ATCC 33270)	<i>P. anaerobius</i> (ATCC 27337)	<i>F. nucleatum</i> (ATCC 25586)	<i>C. albicans</i> (ATCC 10231)
<i>Amoxicillin and Clavulanic acid</i> (10 µg/ml)		13.66	16.31	11.22		
<i>Metronidazole</i> (10 µg/ml)					9.15	
<i>Ciprofloxacin</i> (5µg/ml)	11.25					
<i>Fluconazole</i> (10 µg/ml)						12.68

Arctium lappa root formula showed the minimum inhibitory concentration (MIC) at 6.25 mg/ml for *Enterococcus Faecalis* (ATCC29212), *Pseudomonas aeruginosa* (ATCC27853) and *Candida albicans* (ATCC10231). (Table 5).

Aloe vera gel showed the minimum inhibitory concentration (MIC) at 25 mg/ml for *Peptostreptococcus anaerobius* (ATCC27337) and *Fusobacterium nucleatum* (ATCC25586), 12.5 mg/ml for *Parvimonas micra* (ATCC33270), 6.25 mg/ml for *Pseudomonas aeruginosa* (ATCC27853) and *Candida albicans* (ATCC10231), 3.125 mg/ml for *Enterococcus faecalis* (ATCC29212). (Table 5)

Mix formula of *Arctium lappa* root powder and *Aloe vera* gel showed the minimum inhibitory concentration (MIC) 25 mg/ml for *Peptostreptococcus anaerobius* (ATCC27337), *Fusobacterium nucleatum* (ATCC25586) and *Parvimonas micra* (ATCC33270), 6.25 mg/ml for *Candida albicans* (ATCC10231) and *Pseudomonas aeruginosa* (ATCC27853), 3.125 mg/ml for *Enterococcus faecalis* (ATCC29212) (Table 5).

The lowest value of the minimum inhibitory concentration in case of the mix formula was found for *Enterococcus faecalis* (ATCC29212) (Table 5).

In order to compare specific antibiotics and antifungals, corresponding to the Clinical and Laboratory Standards Institute, minimum inhibitory concentrations for Amoxicillin and Clavulanic acid, Metronidazole, Ciprofloxacin and Fluconazole were determined (Table 6).

Table 5. Minimum Inhibitory Concentration (MIC) for plant extracts (mg/ml.)

Samples	<i>P. aeruginosa</i> (ATCC 27853)	<i>E. faecalis</i> (ATCC 29212)	<i>P. micra</i> (ATCC 33270)	<i>P. anaerobius</i> (ATCC 27337)	<i>F. nucleatum</i> (ATCC 25586)	<i>C. albicans</i> (ATCC 10231)
<i>Arctium lappa</i> Root extract (mg/ml)	6.25	6.25	100	100	100	6.25
<i>Aloe barbadensis</i> Miller gel extract (mg/ml)	6.25	3.125	12.5	25	25	6.25
Mix (mg/ml)	6.25	3.125	25	25	25	6.25

Table 6. Minimum Inhibitory Concentration (MIC) for standard CLSI medications (mg/ml.)

Samples	<i>P. aeruginosa</i> (ATCC 27853)	<i>E. faecalis</i> (ATCC 29212)	<i>P. micra</i> (ATCC 33270)	<i>P. anaerobius</i> (ATCC 27337)	<i>F. nucleatum</i> (ATCC 25586)	<i>C. albicans</i> (ATCC 10231)
<i>Amoxicillin and Clavulanic acid</i> (µg/ml)		1.56	3.125	3.125		
<i>Metronidazole</i> (µg/ml)					6.25	
<i>Ciprofloxacin</i> (µg/ml)	0.19					
<i>Fluconazole</i> (mg/ml)						12.5

Arctium lappa root formula showed the minimum bactericidal concentration (MBC) at 12.5 mg/ml for *Enterococcus Faecalis* (ATCC29212), *Pseudomonas Aeruginosa* (ATCC27853) and *Candida Albicans* (ATCC10231) (Table 7).

Aloe vera gel showed the minimum bactericidal concentration (MBC) at 6.25 mg/ml for *Enterococcus faecalis* (ATCC29212), 12.5 mg/ml for *Pseudomonas aeruginosa* (ATCC27853) and *Candida albicans* (ATCC10231), 50 mg/ml for *Peptostreptococcus anaerobius* (ATCC27337) and *Fusobacterium nucleatum* (ATCC25586) and 25 mg/ml for *Parvinomas micra* (ATCC33270) (Table 7).

Mix formula of *Arctium lappa* root powder and *Aloe vera* gel showed the minimum bactericidal concentration (MBC) 6.25 mg/ml for *Enterococcus faecalis* (ATCC29212), 12.5 mg/ml for *Candida albicans* (ATCC10231) and *Pseudomonas aeruginosa* (ATCC27853), and 50 mg/ml for *Parvinomas micra* (ATCC33270), *Peptostreptococcus anaerobius* (ATCC27337) and *Fusobacterium nucleatum* (ATCC25586) (Table 7).

The lowest value of the minimum bactericidal concentration (MBC) in case of the mix formula was found for *Enterococcus faecalis* (ATCC29212) (Table 7).

Table 7. Minimum bactericidal concentration (MBC) for plant extracts (mg/ml.)

Samples	<i>P. aeruginosa</i> (ATCC 27853)	<i>E. faecalis</i> (ATCC 29212)	<i>P. micra</i> (ATCC 33270)	<i>P. anaerobius</i> (ATCC 27337)	<i>F. nucleatum</i> (ATCC 25586)	<i>C. albicans</i> (ATCC 10231)
<i>Arctium lappa</i> root extract (mg/ml)	12.5	12.5	100	100	100	12.5
<i>Aloe barbadensis</i> Miller gel extract (mg/ml)	12.5	6.25	25	50	50	12.5
Mix (mg/ml)	12.5	6.25	50	50	50	12.5

For the comparison with specific antibiotics and antifungals, corresponding to the Clinical and Laboratory Standards Institute, minimum bactericidal concentrations for Amoxicillin and Clavulanic acid, Metronidazole, Ciprofloxacin and Fluconazole were determined (Table 8).

The working protocol for the root canal therapy follows different steps, all of them converging to a fundamental goal, the complete removal of the whole population of microorganisms. Some bacteria, like *Enterococcus faecalis*, or yeasts, like *Candida albicans* are resistant to the cleaning and shaping of the root canal and also to different intracanal medications. Given these shortcomings, plant-based alternatives are being increasingly studied.

Table 8. Minimum bactericidal concentration (MBC) for standard CLSI medications (mg/ml.)

Samples	<i>P. aeruginosa</i> (ATCC 27853)	<i>E. faecalis</i> (ATCC 29212)	<i>P. micra</i> (ATCC 33270)	<i>P. anaerobius</i> (ATCC 27337)	<i>F. nucleatum</i> (ATCC 25586)	<i>C. albicans</i> (ATCC 10231)
<i>Amoxicillin and Clavulanic acid</i> (10µg/ml)		3.125	6.25	6.25		
<i>Metronidazole</i> (µg/ml)					12.5	
<i>Ciprofloxacin</i> (µg/ml)	0.38					
<i>Fluconazole</i> (mg/ml)						12.5

Although the culture and growth of anaerobic endodontic microorganisms represents a challenge for every researcher, the study of a number of six different bacterial strains adds value to the previously conducted studies [62].

The endodontic environment is a selective system, with preferences for certain species of microorganisms compared to others. Most bacteria are anaerobic or facultative anaerobic microorganisms and their sensitivity to the presence of oxygen varies by species. Progress in microbiological techniques allowed a series of microorganisms associated with endodontic to be identified.

Representative endodontic microorganisms such as *Enterococcus faecalis*, *Fusobacterium nucleatum*, *Peptostreptococcus anaerobius*, *Parvimonas micra*, *Pseudomonas aeruginosa* and *Candida albicans* were used for the antimicrobial and antifungal assay.

The mix extract of *Arctium lappa* root powder and *Aloe vera* gel demonstrated its highest efficiency against *Candida Albicans*, followed by *Enterococcus faecalis*.

Candida albicans is the predominant yeast identified and isolated from the infected root canal. It can adapt to different environments and can adhere to many types of surfaces. It can manifest a strong virulence expression and different morphologic varieties [63]. Research proved that *Candida albicans* is present in a higher percentage in reinfected or retreated root canals, compared to primary endodontic infections and can therefore play a role in the etiology of periapical disease [64]. This study demonstrates a very high efficiency of the mix extract of *Arctium lappa* root powder and *Aloe vera* gel against *Candida albicans*. The inhibition zone of the mix extract (11.62mm) is very close to the inhibition zone of Fluconazole (12.68mm), the specific antifungal medication given by the Clinical and Laboratory Standards Institute in 2015.

Enterococcus faecalis is known as the most rebel endodontic microorganism and is often resistant to intracanal medication [65]. It is frequently the only bacterial species recovered from the previously treated root canals and is able to survive in the root canal on its own, as a single microorganism [66][67][68].

This study showed a good efficiency of the mix extract of *Arctium lappa* root powder and *Aloe vera* gel against *Enterococcus faecalis*, given that it is used in the correct dosage and at the correct minimal inhibitory and bactericidal concentration. The inhibition zone of the mix extract is 5.69 mm, compared to 12.68mm, corresponding to the inhibition zone of Amoxicillin and Clavulanic acid, the specific medication given by the Clinical and Laboratory Standards Institute in 2015.

A medium sensitivity to this formula exhibited *Pseudomonas aeruginosa*, *Parvimonas micra*, *Peptostreptococcus anaerobius* and *Fusobacterium nucleatum*.

Fusobacterium nucleatum and *Pseudomonas aeruginosa* are common microorganisms found in the roots of teeth with periapical radiolucency [69]. Previous studies indicated that *Fusobacterium nucleatum* is responsible for the inflammatory and painful episodes between treatment sessions, when the tooth is left open for drainage [70].

Peptostreptococcus anaerobius and *Parvimonas micra* are Gram-positive anaerobic cocci. The organisms are found in the commensal human flora but also in various infections, like endocarditis or infections of the gastrointestinal and genitourinary tracts [71] [72]. They are known for their implication in the root canal infections but also for the difficulty of their culture and growth [73].

The use of the mix gel at its correct inhibitory and bactericidal concentration diminishes the differences between the inhibition zones of the mix extract, compared to the specific antibiotics or antifungals, corresponding to the Clinical and Laboratory Standards Institute for each microorganism.

The valuable aspects brought by the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) validate the antimicrobial and antifungal effects of the mix extract of *Arctium lappa* root powder and *Aloe vera* gel.

CONCLUSIONS

In the present study we investigated an experimental substance, based on *Arctium lappa* root powder extract and *Aloe vera* gel, in relation with its content in bioactive compounds and its antibacterial and antifungal activity. Regarding the antimicrobial assay, the plant extract presented the highest activity against *Candida albicans*, followed by *Enterococcus faecalis*, the most resistant microorganisms in the root canal system. Our results, correlated with previous studies, show that bioactive compounds like chlorogenic acid, for *Arctium lappa* and aloe-emodin for *Aloe vera* are responsible for the efficiency of the extract against aerobic and anaerobic bacteria and as well against yeasts. The mix extract is also efficient against a series of other endodontic microorganisms, like *Pseudomonas aeruginosa*, *Parvimonas micra*, *Peptostreptococcus anaerobius* and *Fusobacterium nucleatum*. The use of a plant based gel as an auxiliary

method or even as an alternative to the classic root canal disinfection protocol is a step forward in the continuous attempt of the practitioners to obtain a sterilised root canal. Obviously, further and extended studies are needed, in order to define the mechanisms of in vivo antibacterial and antifungal activity .

EXPERIMENTAL SECTION

HPLC/ESI(+)-MS assay

Sample preparation for the injection in the HPLC system. The extraction of the phenolic compounds from the root of *Arctium lappa* was carried out as follows: 2 g of the sample were placed in a round bottom flask, along with 15 ml of 70% ethanol. The extraction was performed in reflux conditions, at 90° C for 2 hours. The solvent was filtered and the residue was extracted a further two times. The extract was combined and evaporated under reduced pressure, to a total volume of 15 ml, in order to obtain the necessary concentration. The sample was filtered through 0,45 µm nylon filter, followed by the injection in the HPLC system.

Aloe vera gel was extracted in 70% ethanol, at a rate of 1/1 (v / v). The sample was filtered through 0,45 µm nylon filter and followed by the injection in the HPLC system.

HPLC/ESI(+)-MS method. For this procedure we used the Agilent 1200 Series HPLC system. The model was equipped with a quaternary pump delivery system, a solvent degasser, an autosampler and a UV-Vis detector, supplied with photodiode and coupled with a singlequadrupole mass detector (MS), Agilent 6110 model (Agilent Technologies, Chelmsford, MA, USA).

We carried out the separation of the phenolic compounds on an Eclipse XDB C18 column, dimensions 4.6 x 150 mm, 5 µm, from Agilent Technologies. The mobile phases consisted in: A- water + 0.1 % acetic acid and B- acetonitrile + 0.1 % acetic acid, with the following gradient evolution: min. 0-2: solvent B 5%, min. 2-18: solvent B 40 %, min. 18-20: solvent B 90 %, min 20-24: solvent B 90 %, min. 24-25: solvent B 5%. The process was carried out for 30 minutes at a temperature of 25° C and a rate of 0.5 ml / min.

We monitored the chromatograms at 280 and 340 nm. We identified the compounds based on their retention times and UV-VIS spectra and performed comparisons with the chlorogenic acid, as a single external standard, purchased from Sigma-Aldrich, Darmstadt (Germany) and with previous published data. Accordingly, the samples were analysed by HPLC/ESI (+)-MS.

We obtained the mass spectrometric data by using the positive ionization mode. For the measurements we used following parameters: ion spray voltage of 3000V, capillary temperature of 300°C, nitrogen flow rate 8 l min., m/z:100-1000, full-scan. Data acquisition and interpretation of results was done using ChemStation software, Agilent Technologies, Chelmsford, MA, USA.

Antibacterial and antifungal activity. For the bioassay we used five bacterial strains and a yeast: one aerobic bacteria (*Pseudomonas aeruginosa*-ATCC27853), one aerotolerant anaerobic bacteria (*Enterococcus faecalis*-ATCC29212), three anaerobic bacteria (*Parvinomas micra*-ATCC33270, *Peptostreptococcus anaerobius*-ATCC27337, *Fusobacterium nucleatum*-ATCC25586) and a yeast, *Candida albicans* (ATCC10231). The microbial strains were purchased from Microbiologics, Saint Cloud, Minnesota, USA. All tested microorganisms were at the fourth passage from the American Type Culture Collection, as described in their Quality Control certificates. We cultured the bacteria on Muller-Hinton Agar (BioMerieux, Lyon, France) for aerobic microorganisms, on Columbia Agar (BioMerieux, Lyon, France) for anaerobic microorganisms and on Sabouraud Agar (BioMerieux, Lyon, France) for yeast. Cultures were stored at 4°C and subcultured once a month.

For the disc diffusion assay, we used the following plant extracts: 100 mg of *Arctium lappa* root powder, suspended in 1 ml distilled water, mixed for 8 hours and filtered by 0.45 µm Millipore filter, 100 ml of *Aloe vera* gel extract and 1:1 *Arctium lappa* root extract and *Aloe vera* gel mixture. We carried out the primary antimicrobial test screening by disc diffusion, using 100 µl of suspension containing 10⁸ CFU/ml of bacteria and 10⁶ CFU/ml of *Candida albicans*, spread evenly on the surface of the Muller–Hinton Agar, Columbia Agar, respectively Sabouraud Agar plates. Sterile 6 mm discs were used and processed, in triplicates, to contain 20 µl of the extract; these discs were then placed on the inoculated agar. For each studied microorganism we used specific antibiotics as following: *Amoxicillin* and *Clavulanic acid* for *Enterococcus faecalis*, *Peptostreptococcus anaerobius* and *Parvimonas micra*, *Metronidazole* for *Fusobacterium nucleatum*, *Ciprofloxacin* for *Pseudomonas aeruginosa* and *Fluconazole* for *Candida albicans*. For aerobic bacteria, the inoculated plates were incubated for 24 h at 37°C, for anaerobic bacteria for 48 h at 37°C in CO₂ gas bags (BioMerieux, Lyon, France) and for *Candida albicans* the inoculated plates were incubated for 2-3 days at 30°C. Clear inhibition zones around discs indicated the presence of antimicrobial activity. For high fidelity of the results, each assay was repeated three times.

Microdilution method. We used the modified microdilution technique to evaluate the antimicrobial and antifungal activity. We cultured the bacterial species overnight, at 37°C in Muller-Hinton Broth (BioMerieux, Lyon, France) for aerobics and yeast and on Thioglycollate Broth with Resazurin (BioMerieux, Lyon, France) for anaerobics. We adjusted the bacterial cell suspensions with sterile saline solution, to a concentration of approximately 3X 10⁵ CFU/ml in a final volume of 100 µl per well. We stored the inoculum at +4°C for further use. We cultured dilutions of the inoculum on solid Muller–Hinton Agar (BioMerieux, Lyon, France), and Columbia Agar (BioMerieux, Lyon, France) for bacteria, to verify the absence of contamination and to check the validity of the inoculum. We determined the minimum inhibitory concentrations (MICs) by a serial dilution technique, using 96-well microtitre plates. Different solvent dilutions of ethanol were carried out: methanol extracts were carried out over the wells containing 100 µl of Muller-Hinton Broth or Thioglycollate broth

with Resazurin and afterwards, 10 µl of inoculum was added to all the wells. The microplates were incubated for 24–48 h at 37°C. We detected the minimum inhibitory concentration of the samples following the addition of 20 µl (0.2 mg/ml) of Resazurin solution to each well, and we incubated the plates for 2 h at 37°C. In general, a change from blue to pink indicates reduction of Resazurin and therefore bacterial growth. The minimum inhibitory concentration was defined as the lowest drug concentration that prevented this colour change.

We determined the minimum bactericidal concentrations (MBCs) by serial subcultivation of a 2 µl into microtitre plates, containing 100 µl of broth per well and further incubation for 48 h at 37°C. The lowest concentration with no visible growth was defined as minimum bactericidal concentration, indicating 99.5% killing of the original inoculum. As a positive control for bacterial growth we used Streptomycin (Sigma P 7794), 0.05–3 mg/ml.

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